

PRE-CLINICAL RESEARCH

Phenyl- α -tert-butyl-nitrone and Benzonidazole Treatment Controlled the Mitochondrial Oxidative Stress and Evolution of Cardiomyopathy in Chronic Chagasic Rats

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Objectives

The purpose of this study was to determine the pathological importance of oxidative stress-induced injurious processes in chagasic heart dysfunction.

Background

Trypanosoma cruzi-induced inflammatory pathology and a feedback cycle of mitochondrial dysfunction and oxidative stress may contribute to Chagas disease.

Methods

Sprague-Dawley rats were infected with *T. cruzi* and treated with phenyl- α -tert-butyl-nitrone (PBN), an anti-oxidant, and/or benzonidazole (BZ), an antiparasitic agent. We monitored myocardial parasite burden, oxidative adducts, mitochondrial complex activities, respiration, and adenosine triphosphate synthesis rates, and inflammatory and cardiac remodeling responses during disease development. The cardiac hemodynamics was determined for all rats.

Results

Benzonidazole (not PBN) decreased the parasite persistence and immune adverse events (proinflammatory cytokine expression, β -nicotinamide adenine dinucleotide phosphate oxidase and myeloperoxidase activities, and inflammatory infiltrate) in chronically infected hearts. PBN \pm BZ (not BZ alone) decreased the mitochondrial reactive oxygen species level, oxidative adducts (malonyldialdehyde, 4-hydroxynonenal, carbonyls), hypertrophic gene expression (atrial natriuretic peptide, B-type natriuretic peptide, α -skeletal actin), and collagen deposition and preserved the respiratory chain efficiency and energy status in chronically infected hearts. Subsequently, LV dysfunction was prevented in PBN \pm BZ-treated chagasic rats.

Conclusions

BZ treatment after the acute stage decreased the parasite persistence and inflammatory pathology. Yet, oxidative adducts, mitochondrial dysfunction, and remodeling responses persisted and contributed to declining cardiac function in chagasic rats. Combination treatment (PBN + BZ) was beneficial in arresting the *T. cruzi*-induced inflammatory and oxidative pathology and chronic heart failure in chagasic rats. (J Am Coll Cardiol 2010;55:2499–508) © 2010 by the American College of Cardiology Foundation

Chagas disease is an emerging parasitic disease in developed countries. In endemic countries, ~16 to 18 million people are infected with *Trypanosoma cruzi* and at risk of disease development (1). In most patients, acute infection results in nonspecific clinical symptoms. Several years after that, 30% to 40% of seropositive individuals develop progressive cha-

gasic cardiomyopathy with ventricular dilation, arrhythmia, and contractile dysfunction, leading to cardiac arrest and patient's death (2).

Several lines of evidence establish that a low level of parasites remains in the host during the chronic disease phase (3). Direct detection of parasites in autopsy specimens and myocardial biopsy specimens has been shown by immunohistochemical and immunofluorescence techniques (4) and polymerase chain reaction (PCR) (5). The reactivation of acute infection and parasitemia has been noted after immunosuppression due to acquired immunodeficiency syndrome (6) or drug therapy (7). Blood transfusion and transplantation of infected organs obtained from asymptomatic individuals are major causes of *T. cruzi* transmission

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Abbreviations and Acronyms

α_{sk} actin	= α -skeletal actin
ANOVA	= analysis of variance
ANP	= atrial natriuretic peptide
ATP	= adenosine triphosphate
BNP	= B-type natriuretic peptide
BZ	= benzonidazole
HNE	= 4-hydroxynonenal
LV	= left ventricular
MDA	= malonyldialdehyde
MPO	= myeloperoxidase
NOX	= β -nicotinamide adenine dinucleotide phosphate oxidase
PBN	= phenyl- α -tert-butyl nitron
PCR	= polymerase chain reaction
P-V	= pressure-volume
pyr/mal	= pyruvate/malate
ROS	= reactive oxygen species
XOD	= xanthine oxidase

in nonendemic countries (8). These studies indicated that parasite persistence results in consistent activation of inflammatory responses and the development and/or propagation of pathological lesions in the heart.

We and others (9) have shown that chagasic myocardia sustain oxidative injuries. Phagocytes (macrophages and neutrophils), activated to control *T. cruzi* infection, are a major source of oxidative stress in the acutely infected host. Further, myocardial oxidative stress is enhanced due to increased mitochondrial production of reactive oxygen species (ROS) during Chagas disease (10). A compromised antioxidant status in *T. cruzi*-infected experimental animals (11) was associated with enhanced oxidative modification of cellular (11,12) and mitochondrial proteins and lipids (13). The plasma and intraerythrocyte levels of glutathione and glutathione peroxidase are decreased (14), and glutathione disulfide and malonyldialdehyde (MDA) contents

increased (15) in human chagasic patients. These studies indicated that oxidative injurious processes are of pathological significance in Chagas disease.

In this study, we aimed to evaluate the relative pathological importance of parasite persistence and oxidative injurious processes in the progression of Chagas disease and cardiac contractile dysfunction. Sprague-Dawley rats were infected with *T. cruzi* and treated with phenyl- α -tert-butyl nitron (PBN), a nitron-based antioxidant that scavenges a wide variety of free radical species and inhibits free radical generation (16). Some of the infected rats were treated with benzonidazole (BZ), which is currently the treatment of choice for chagasic patients (17). We determined the efficacy of PBN intervention in reducing the generation or the effects of ROS and of BZ in controlling parasite persistence and subsequently evaluated whether these treatments (individually or in combination) were beneficial in preventing progressive chagasic pathology and cardiac dysfunction in chronically infected rats.

Methods

Details are provided in the Online Appendix.

Animals and parasites. Sprague-Dawley rats (4 to 5 weeks old, Harlan Laboratories, Indianapolis, Indiana) were ac-

customed to animal facility for 1 month and then infected with *T. cruzi* (SylvioX10/4, 50,000 trypomastigotes per rat, intraperitoneally). Rats were given PBN (1.3 mM, beginning day 0 throughout the course of infection) and/or BZ (0.7 mM, beginning day 40 post-infection, for 3 weeks) in drinking water. Tissue parasite burden was determined by traditional and real-time PCR using *T. cruzi* 18SrDNA-specific oligonucleotides (18). The institutional Animal Care and Use Committee approved all animal experiments.

ROS and adenosine triphosphate (ATP). The myocardial and mitochondrial levels of ROS were determined using dihydroethidium and amplex red/horseradish peroxidase fluorescent probes (19). Cryostat tissue sections were stained with dihydroethidium to detect in situ ROS. The ATP level in tissue homogenates and isolated mitochondria was determined by a luciferin/luciferase bioluminescence method (19). Mitochondria were energized with pyr/mal (pyruvate/malate) and succinate to monitor the rates of ROS and ATP production.

Lipid and protein oxidation. The MDA level was determined by a thiobarbituric acid reactive substances assay (20). Protein carbonyls were derivatized with 2,4-dinitrophenylhydrazine and estimated by enzyme-linked immunosorbent assay (21) using a polyclonal anti-2,4-dinitrophenylhydrazine antibody (Chemicon, Billerica, Massachusetts). Paraffin-embedded tissue sections (5 μ m) were subjected to immunostaining with an anti-4-hydroxynonenal (HNE) antibody (Alpha Diagnostics, San Antonio, Texas), and images analyzed by light microscopy.

Inflammation and tissue pathology. Formalin-fixed myocardial tissue sections were stained with hematoxylin/eosin or Masson's trichrome and scored for myocarditis, fibrosis, and parasitic lesions (22). The enzymatic activities of xanthine oxidase (XOD), β -nicotinamide adenine dinucleotide phosphate oxidase (NOX), and myeloperoxidase (MPO) were monitored by spectrophotometry and/or in-gel catalytic staining.

The myocardial mRNA levels for proinflammatory cytokines and hypertrophy markers were determined by real-time reverse transcriptase PCR (18).

Heart function. Rats were monitored for heart function during the acute (25 to 40 days post-infection) and chronic (>150 days post-infection) stages of infection. After intraperitoneal injection of heparin (1,000 IU/kg) and administration of anesthesia, tracheotomy was performed to facilitate breathing, and a conductance pressure-volume (P-V) catheter (Scisense, Inc., London, Ontario, Canada) was inserted into the left ventricle. Respiration (80 breaths/min, 3 l/min O₂) was controlled through a tracheotomy cannula connected to a pressure-controlled respirator (RSP1002, Kent Scientific, Litchfield, Connecticut). The conductance and pressure signals were digitized, and the volume calibration of the conductance system was performed. Hemodynamic measurements were made using emka cardiac P-V

analysis software (emka TECHNOLOGIES USA, Falls Church, Virginia).

Data analysis. Data were expressed as mean ± SD (9 animals per group, triplicate observations per experiment), analyzed by SPSS software version 14.0 (SPSS, Inc., Chicago, Illinois). Normally distributed data were analyzed by the Student *t* test (comparison of 2 groups) and 1-way analysis of variance (ANOVA) with Tukey's post-hoc test (comparison of multiple groups). Mann-Whitney and Kruskal-Wallis tests were used when data were not normally distributed. Significance is shown by *normal-versus-infected/untreated and #infected/untreated-versus-infected/treated (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

Results

Parasite burden. Semiquantitative PCR showed a significant level of *Tc18SrDNA* signal in the myocardium of acutely and chronically infected rats (Figs. 1A and 1B). The extent of *Tc18SrDNA* amplification was similar in the heart tissue of infected/PBN-treated and infected/untreated rats. In comparison, *Tc18SrDNA*-specific signal was barely detectable in the myocardium of BZ-treated/chronically infected rats. Specificity of the PCR was confirmed by no amplification of *Tc18SrDNA* with

normal rat DNA. Real-time PCR was used to obtain a quantitative measure of parasite burden in infected rats. Our data showed a 15-fold higher parasite burden in acutely infected hearts compared with chronically infected hearts (Fig. 1C). BZ treatment (± PBN) resulted in 55% decrease in *Tc18SrDNA* signal in infected rats (Fig. 1C) (*p* < 0.01 [ANOVA-Tukey's]). These results showed that BZ was effective in controlling the parasite persistence, and PBN alone had no effect on parasite replication and/or survival in the heart.

ROS and oxidative damage. Fluorometric evaluation of ROS showed 72% and 63% increases in H₂O₂ levels in the myocardial homogenates of acutely and chronically infected rats (Fig. 2A) (*p* < 0.01 [*t* test]) that was not controlled in infected/BZ-treated rats. When treated with PBN (± BZ), the enhanced level of H₂O₂ was controlled by 96% in acute myocardium and by 49% to 53% in chronically infected hearts (Fig. 2A) (all *p* values < 0.01 [*t* test/ANOVA-Tukey's]). In situ studies verified these results. We noted a substantial DHE oxidation to ethidium red in the myocardial sections of acutely and chronically infected rats that were not treated or treated with BZ only (Fig. 2B). Infected rats treated with PBN (± BZ) exhibited a significant decrease in ethidium

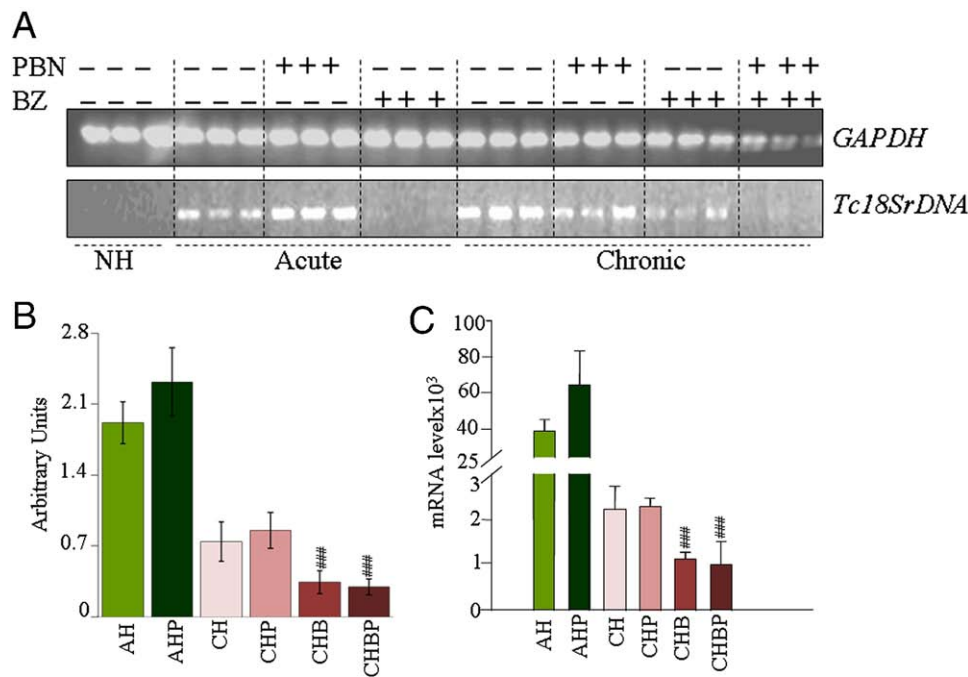
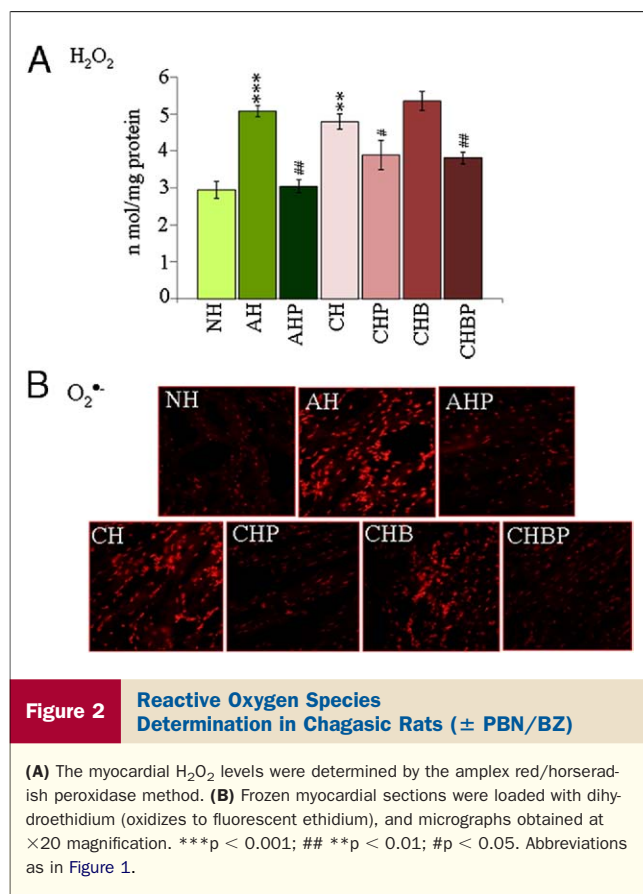


Figure 1 Tissue Burden of *Trypanosoma cruzi* in Infected Rats (± PBN/BZ)

Myocardial total DNA was used as a template, and *T. cruzi* 18SrDNA amplified by PCR (A, B) and real-time polymerase chain reaction (PCR) (C). Densitometric quantification of Tc18SrDNA PCR amplicons (28 cycles), normalized to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is shown in B. Data in all figures are presented as mean ± SD (n = 9; ###*p* < 0.001). AH = acutely infected/untreated (25 days post-infection); AHP = acutely infected/PBN treated; BZ = benzonidazole; CH = chronically infected/untreated (>150 days post-infection); CHP = chronically infected/PBN treated; CHB = chronically infected/BZ treated; CHBP = chronically infected/PBN + BZ treated; NH = normal heart; PBN = phenyl- α -tert-butyl nitron.



fluorescence during the acute and chronic stages of infection and disease development (Fig. 2B).

In agreement with ROS levels, the myocardial contents of MDA and carbonyl adducts were increased by 75% and 189%, respectively, in the acutely infected rats and by 66% and 168%, respectively, in chronically infected rats (Figs. 3A and 3B) ($p < 0.001$ [t test]). The PBN (\pm BZ) treatment resulted in a decrease in myocardial MDA and carbonyl contents by 62% and 77%, respectively, in the acute stage and by 84% to 88% and 100%, respectively, in the chronic stage (all p values < 0.001 [t test/ANOVA-Tukey's]). Rats treated with BZ only exhibited a slight control of MDA (31% decrease) and no decrease in carbonyl adducts compared with infected/untreated rats. Immunostaining showed extensive HNE adducts (intensity score: 2 to 3) in acutely and chronically infected rats and in chronically infected/BZ-treated rats (Fig. 3C). HNE adducts were significantly decreased (intensity score: 0 to 1) in the myocardium of PBN-treated (\pm BZ) rats at both acute and chronic stages of infection and disease development (Fig. 3C). These data (Figs. 2 and 3) showed that PBN (but not BZ) treatment decreased the ROS level and oxidative damage in the myocardium of infected rats.

Mitochondrial dysfunction. Cardiac mitochondria of infected rats exhibited 41% to 43% and 54% to 58% decreases in the CI (reduced nicotinamide adenine dinucleotide-quinone oxidoreductase) and CIII (cytochrome-c oxidoreductase) respiratory complex activities, respectively,

during the course of infection and disease development (Figs. 4A and 4B) ($p < 0.01$ [t test]). The respiratory chain inefficiency was associated with enhanced mitochondrial ROS production. The pyr/mal- and succinate-stimulated mitochondrial ROS production was distinctly increased in infected myocardium (acute: 2-fold and 95%, respectively; chronic: 1.8-fold and 91%, respectively) (Fig. 4C) ($p < 0.001$ [t test]). Treatment with PBN (\pm BZ) resulted in 70% and 43% to 88% improvement in CI activity in acutely and chronically infected rats, respectively (Fig. 4A) and normalization of CIII activity in infected myocardium (Fig. 4B). Subsequently, pyr/mal- and succinate-dependent ROS formation was significantly controlled in PBN-treated (\pm BZ) rats during acute (82% and 96% decrease, respectively) and chronic (57% and 32% decrease, respectively) stages (Fig. 4C) (all p values < 0.001 [t test/ANOVA-Tukey's]). Treatment with BZ alone failed to preserve the respiratory complex activities or avert the increased rate of ROS production in cardiac mitochondria of infected rats.

The pyr/mal- and succinate-dependent mitochondrial rate of ATP synthesis was decreased by 54% to 57% in infected myocardium (Fig. 4D), and associated with 68% to 80% and 52% to 62% decreases in mitochondrial and myocardial ATP levels, respectively (Figs. 4E and 4F). PBN treatment (\pm BZ) improved the pyr/mal- and succinate-dependent mitochondrial ATP production by 49% to 61% in infected hearts (Fig. 4E) (all p values < 0.01 [t test/ANOVA-Tukey's]). Subsequently, mitochondrial and cardiac ATP contents were improved in PBN-treated (\pm BZ) rats by 35% to 46% (Figs. 4E and 4F) (all p values < 0.01 [t test/ANOVA-Tukey's]). Treatment with BZ alone resulted in no significant improvement in mitochondrial ATP synthesis and myocardial ATP levels in infected rats (Figs. 4D to 4F). These data (Fig. 4) demonstrated that PBN (but not BZ) preserved the mitochondrial respiratory chain activity and ATP production and prevented enhanced ROS formation in the myocardium of acutely and chronically infected rats.

BZ kills *T. cruzi* via oxidative stress (23) and thereby may have standby effects on host mitochondria. To determine whether such is the case, we compared myocardial mitochondrial respiration in normal and normal/BZ-treated rats. We observed no significant differences in the rate of substrate-stimulated oxygen consumption in left ventricle slices (in vivo) of normal and normal/BZ-treated rats. Likewise, state 3 respiration and state 3/state 4 ratio of isolated cardiac mitochondria from normal/BZ-treated were not compromised (Online Fig. 1, Online Table 2). Further, BZ-treated/normal rats exhibited no significant increase in MDA and carbonyl adducts (data not shown). Thus, BZ alone did not contribute to mitochondrial dysfunction and oxidative stress in the host myocardium.

Inflammatory pathology. Histological studies showed parasitic foci and intense inflammatory infiltrate composed of mononuclear (macrophages, T cells) and polymorphonuclear (neutrophils) cells in acute myocardium (histological

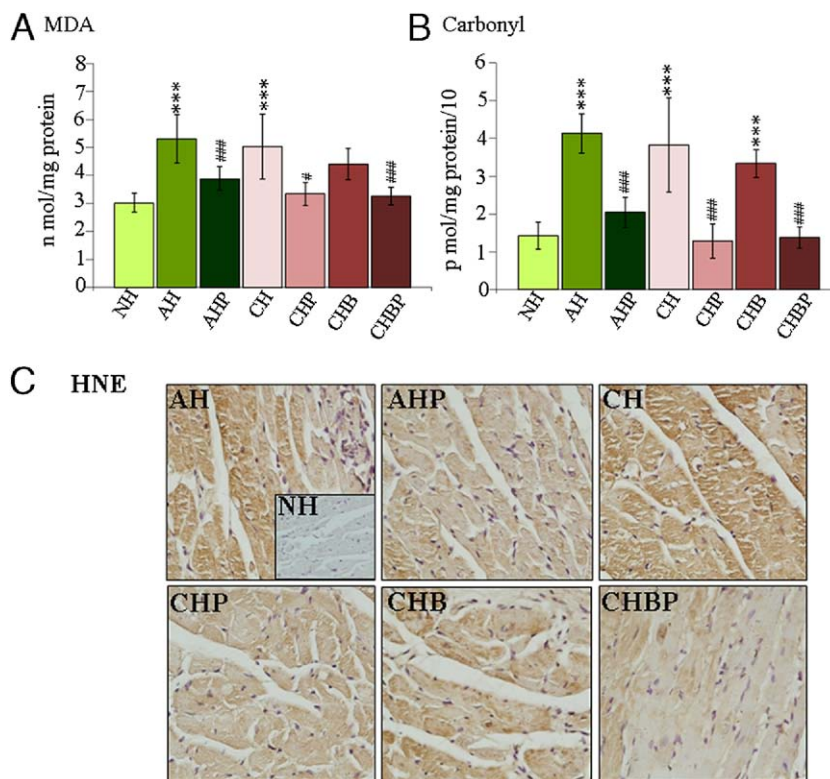


Figure 3 Myocardial Oxidative Damage in *Trypanosoma cruzi*-Infected Rats (\pm PBN/BZ)

(A) Thiobarbituric acid reactive substances assay determination of myocardial malonyldialdehydes (MDA). (B) 2,4-dinitrophenylhydrazine-derivatized carbonyl proteins were measured by enzyme-linked immunosorbent assay. (C) Immunohistochemical staining of heart tissue sections with anti-4-hydroxynonenal (HNE) antibody (magnification $\times 40$). *** ###p < 0.001; #p < 0.05.

score 2 to 4) (Fig. 5A). The inflammatory sequel was moderate and diffused in chronically infected hearts (histological score 1 to 2). The mRNA levels for proinflammatory cytokines interleukin-1 β , interferon gamma, and tumor necrosis factor- α were significantly increased in acutely and chronically infected myocardium (Figs. 5B through 5D; all p values < 0.001 [*t* test]). PBN treatment of infected rats resulted in a moderate decrease in myocardial inflammatory infiltrate (histological score 2 to 3) and marginal to no change in cytokine mRNA levels. In comparison, infected rats treated with BZ (\pm PBN) exhibited a considerable decrease in myocardial inflammatory infiltrate (histological score 0 to 1). Accordingly, the myocardial mRNA levels for interleukin-1 β , interferon gamma, and tumor necrosis factor- α were significantly decreased in chronically infected rats treated with BZ only (100%, 99%, and 89.5% decrease, respectively; p < 0.01 [ANOVA-Tukey's]) or treated with a combination of PBN and BZ (normalized to normal control level; p < 0.01 [ANOVA-Tukey's]) (Fig. 5).

NOX, MPO, and XOD are indicators of macrophage, neutrophil, and endothelial cell activation, respectively. Spectrophotometric studies showed a substantial increase in NOX, MPO, and XOD activities in the infected myocar-

dium (acute: 7.6-, 3.2-, and 43.1-fold increase, respectively; chronic: 110%, 2.2-fold, and 108% increase, respectively) (Figs. 5E to 5G) (p < 0.01 [*t* test]). PBN treatment (\pm BZ) led to a significant control of NOX and MPO activities in the acute (60% to 70% and 63%, respectively, all p values < 0.01 [*t* test]) and chronic (55% to 95% and 58% to 71%, respectively; all p values < 0.01 [ANOVA-Tukey's]) hearts. Likewise, BZ treatment was effective and resulted in 16% to 95% decrease in NOX and 25% to 71% decrease in MPO activities, respectively, in chronically infected myocardium (p < 0.01 [ANOVA-Tukey's]). PBN and BZ treatments (individually or in combination) did not alter the XOD activity in infected rats (Fig. 5G). In-gel catalytic staining assays validated the spectrophotometric results and showed that PBN and BZ (individually or in combination) controlled the enhanced activities of NOX and MPO in infected myocardium (Online Fig. 2). These data demonstrated that the parasite-dependent infiltration of inflammatory infiltrate and cytokine expression in the heart were significantly controlled by BZ. PBN was primarily effective in limiting the inflammatory oxidative stress in chagasic hearts as evidenced by a decrease in NOX and MPO activities.

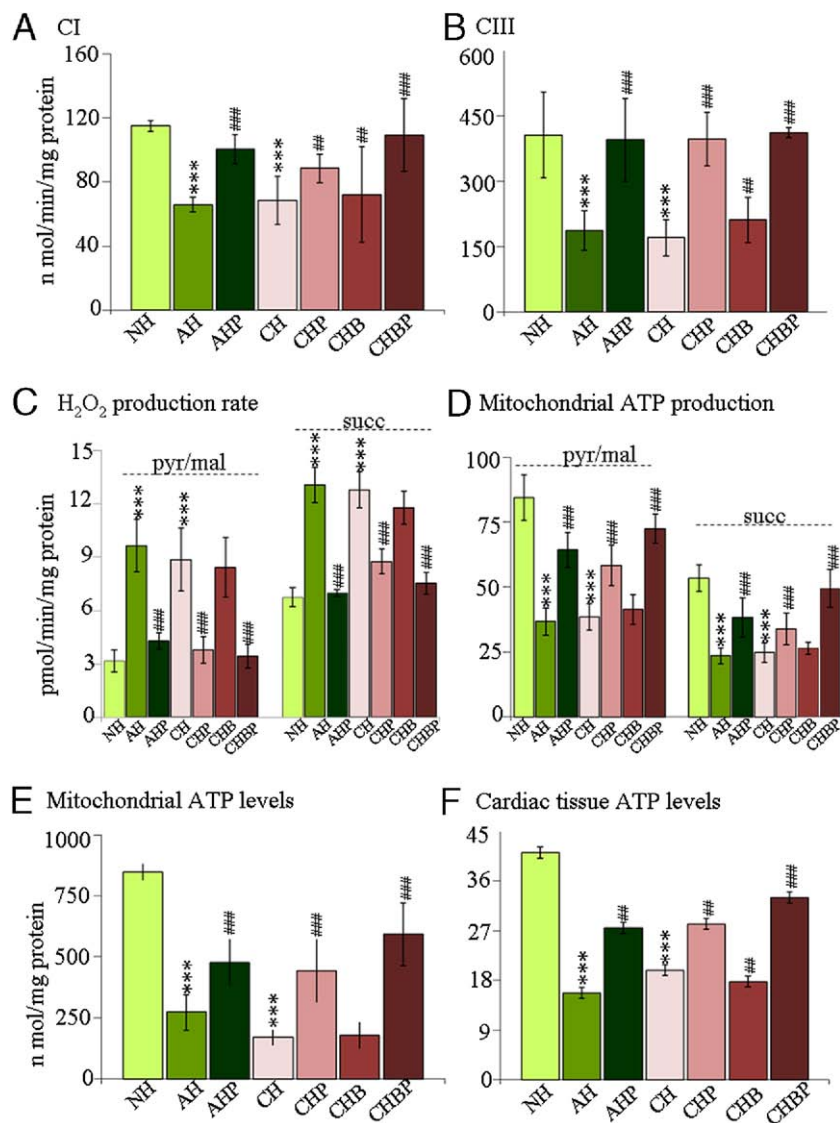


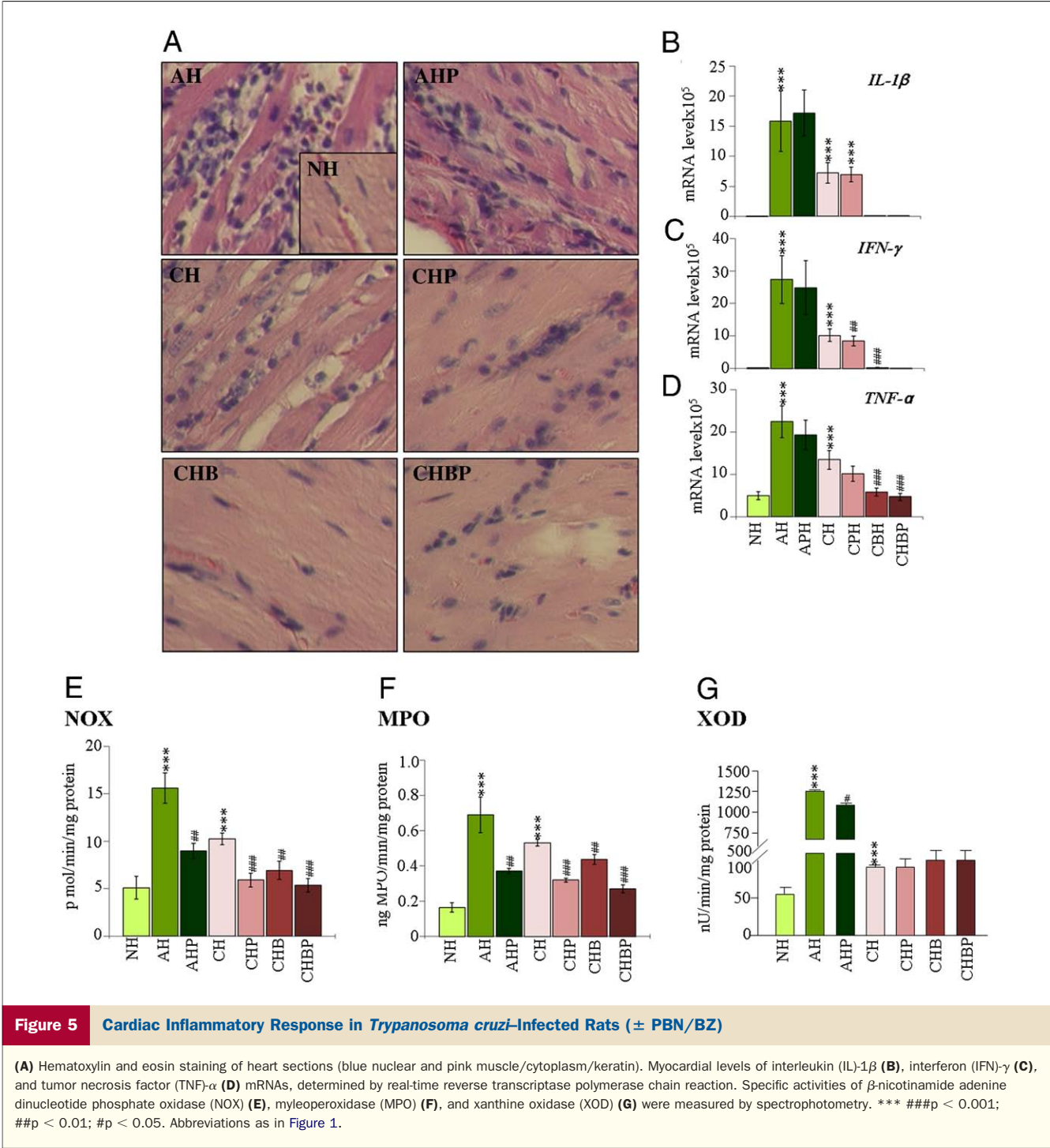
Figure 4 Mitochondrial Functional Analysis in Chagasic Rats (± PBN/BZ)

Spectrophotometric measurement of complex I (A) and complex III (B) activities in isolated cardiac mitochondria. The rate of H₂O₂ (C) and adenosine triphosphate (ATP) (D) production was monitored in pyruvate/malate (pyr/mal)- and succinate (succ)-energized isolated cardiac mitochondria. The mitochondrial (E) and myocardial (F) ATP levels were determined by a luciferin/luciferase bioluminescence method. *** ###p < 0.001; ##p < 0.01. Abbreviations as in Figure 1.

Cardiac remodeling. We performed Masson's trichrome staining of heart sections to detect collagen deposition. In acutely infected rats, scattered foci of myocyte necrosis associated with inflammatory cells were visible; however, no significant evidence of fibrosis was detectable (Fig. 6A). Histological observation of extensive collagen deposition in chronically infected rats (histological score 2 to 4) was associated with a 10.4-, 4.4-, and 11.2-fold increase in ANP, BNP, and α_{sk} actin mRNAs, respectively (Figs. 6B to 6D) (all p values <0.001 [*t* test]). We observed a moderate decrease (histological score 2 to 3) in collagen deposition (Fig. 6A), and a distinct decrease in the enhanced ANP, BNP, and α_{sk} actin mRNA levels in infected rats treated

with PBN (82%, 77%, and 92%, respectively) or PBN/BZ (79.7%, 72.5%, and 74.8%, respectively) (Figs. 6B to 6D) (all p values <0.00 [*t* test/ANOVA-Tukey's]). Treatment with BZ alone resulted in a marginal decrease in collagen deposition (histological score 3 to 4) and BNP and α_{sk} actin mRNAs in chronically infected myocardium. These results suggested that adjunct therapy with PBN and BZ was effective in limiting cardiac remodeling responses in chagasic rats.

Hemodynamic measurements. P-V loops were analyzed at steady state and after inferior vena cava occlusion (Online Table 3). Baseline measurements showed no significant differences in the hemodynamics of normal and acute rats



(data not shown). The pressure shifts of the P-V loops from baseline (mm Hg: normal, 82.3; chronically infected, 48.1) indicated a decrease in cardiac contractility in the chronic stage. The left ventricular (LV) $+dp/dt_{max}$ (peak rate of pressure rise), $-dp/dt_{max}$ (peak rate of pressure decline), stroke volume (blood volume pumped with each beat), cardiac output (blood volume pumped per minute), ejection fraction (fraction of blood ejected/contraction), and heart rate were decreased by 28%, 43%, 13%, 46%, 34%, and 38%,

respectively, in chronically infected rats (all p values <0.01 [Mann-Whitney]). Notable elevation in end-systolic volume and end-diastolic volume occurred in chronically infected hearts with 174% and 44% increase, respectively, compared with controls. No significant gain in cardiac function was observed in chronically infected/BZ-treated rats. In comparison, a gain in cardiac function with PBN treatment was evident by a significant elevation of $+dp/dt_{max}$, $-dp/dt_{max}$, stroke volume, cardiac output, ejection fraction, and heart rate in

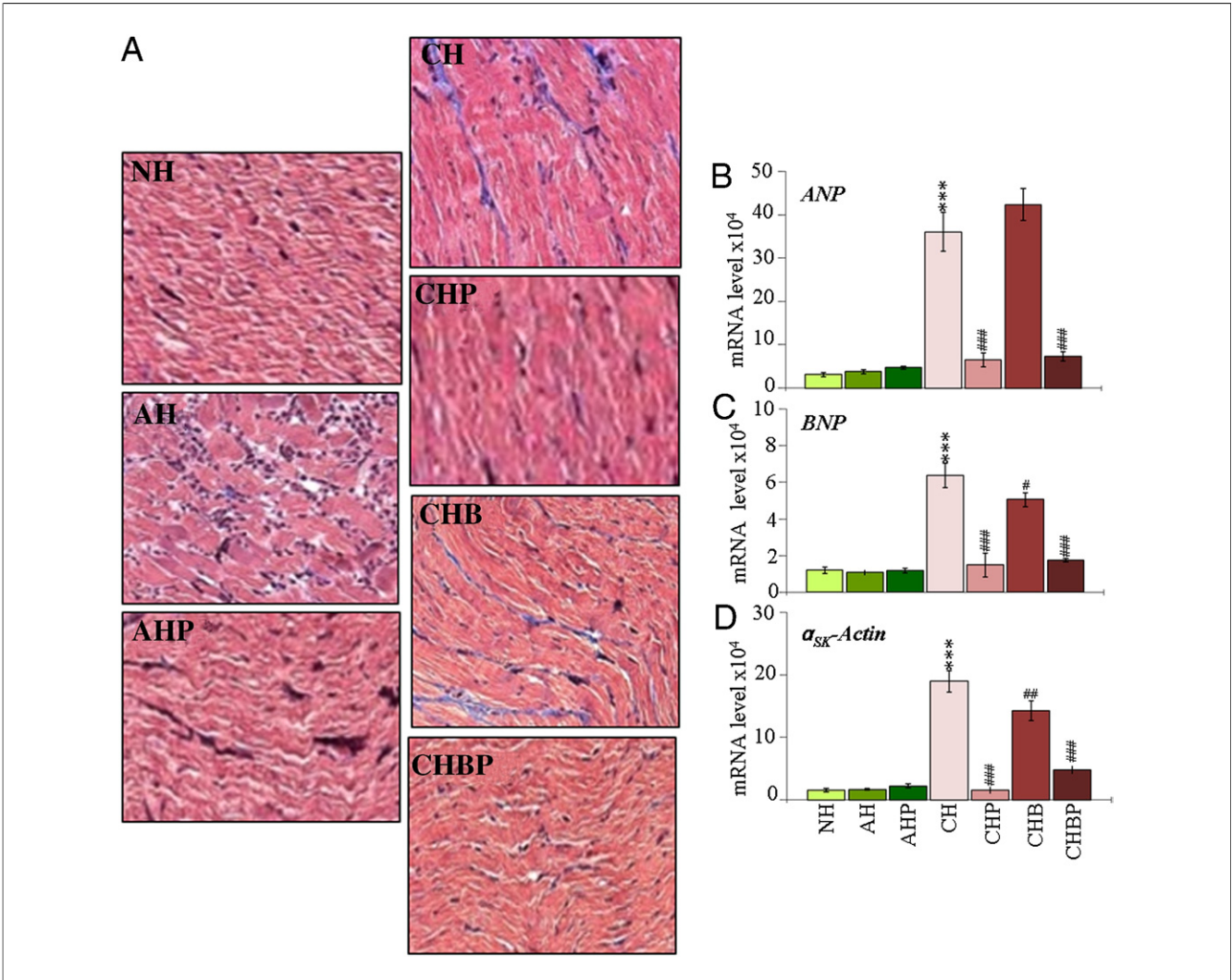


Figure 6 Myocardial Remodeling in *Trypanosoma cruzi*-Infected Rats (\pm PBN/BZ)

(A) Masson's trichrome staining of heart sections (blue collagen, red muscle/cytoplasm). Real-time reverse transcriptase polymerase chain reaction measurement of myocardial mRNA levels for atrial natriuretic peptide (ANP) (B), brain-natriuretic-peptide (BNP) (C), and α -skeletal actin (α_{sk} -Actin) (D). *** ###p < 0.001; #p < 0.05; ##p < 0.01. Abbreviations as in Figure 1.

chronically infected, PBN-treated rats (48%, 59%, 26%, 70%, 93%, and 87%, respectively; all p values <0.01 [Kruskal-Wallis]) and PBN/BZ-treated rats (52%, 53%, 100%, 96%, 91%, and 90%, respectively; all p values <0.001 [Kruskal-Wallis]). Subsequently, end-systolic volume and end-diastolic volume elevation noted in chronic chagasic hearts was normalized to control level by PBN (\pm BZ) treatment (all p values <0.01 [Kruskal-Wallis]).

P-V loops with inferior vena cava occlusion showed that the end-systolic P-V relationship (indicator of systolic performance) was decreased by 48% and the end-diastolic P-V relationship (indicator of diastolic stiffness) was increased by 68% in chronically infected rats, and no benefit was afforded by BZ treatment. PBN treatment (\pm BZ) was beneficial as evidenced by <10% decrease in end-systolic P-V relationship and <15% increase in end-diastolic P-V relationship compared with normal controls. Thus, hemo-

dynamic results showed that PBN markedly prevented the chagasic cardiac dysfunction.

Discussion

In this study, we demonstrate that decreasing the oxidative stress-induced alterations in myocardial structure, gene expression, and mitochondrial function is beneficial in preventing the chronic evolution of cardiac dysfunction in Chagas disease. We also provide molecular and biochemical evidence of the inefficacy of BZ during chronic Chagas disease.

We used a rodent model of infection and disease development that exhibited the classic hallmarks of chronic Chagas disease. Infected rats showed a high level of acute parasite burden (Fig. 1), resulting in extensive infiltration of inflammatory infiltrate and expression of proinflammatory

cytokines in the heart (Fig. 5). During the chronic stage, diffused inflammatory response persisted in the heart. Cardiac remodeling in chronically infected rats was evidenced by an increased expression of hypertrophy markers (ANP, BNP, and α_{sk} actin) and collagen deposition (Fig. 6). Subsequently, ventricular performance was compromised, as evidenced by 34% to 46% decrease in cardiac output and ejection fraction and alterations in other parameters proposed as a fair index of contractility (Online Table 3) (24). The baseline hemodynamics of chronically infected rats, obtained with the P-V loops, were in accordance with the results from human chagasic patients (25).

A systematic review of the literature indicates that the pathogenesis of Chagas disease is dependent on a low-grade, systemic infection with documented immune system adverse reactions (discussed in Sack et al. [3]). Only acutely infected patients, irrespective of their ages, are shown to respond to treatment with an antiparasite drug (BZ) and be cured, defined by the control of acute parasitemia and myocarditis (17). No convincing evidence exists demonstrating the efficacy of BZ in chronically infected patients. Further, it is not known whether drug therapies are ineffective in chronically infected patients because either BZ fails to kill tissue parasites or other host events persist independent of parasites and result in the evolution of chronic heart failure. To investigate this, we examined in our experimental model the effect of BZ treatment on parasites and host events. Our data showed that oral delivery of BZ for 3 weeks during an indeterminate phase precluded the tissue parasite persistence and associated inflammation in chronically infected hearts (Figs. 1 and 5). Activated neutrophils and macrophages express NOX, inducible nitric oxide synthase, and MPO and participate in parasite control through a release of cytotoxic ROS, nitric oxide, and HOCl, respectively (26). Our observation of a decrease in NOX and MPO activities (Fig. 5, Online Fig. 2) in BZ-treated chronically infected rats provided further evidence that antiparasite therapy was effective in averting the parasite-mediated inflammatory pathology. Nonetheless, BZ-treated/chronically infected rats exhibited cardiac remodeling (Fig. 6) and deterioration of ventricular contractility (Online Table 3) comparable to that exhibited by untreated/chronically infected rats. These data provide molecular evidence that a lack of control of chronic Chagas disease by BZ is not due to its inefficacy in controlling parasite persistence and parasite-dependent inflammatory responses. Importantly, the observations in BZ-treated rats of consistent oxidative stress-induced myocardial adducts (Fig. 3) and mitochondrial inefficiency (Fig. 4) strongly indicate a role of oxidative stress and mitochondrial/cellular injuries in LV dysfunction during progressive Chagas disease. The mitochondrial production of ROS was significantly enhanced in BZ-treated and untreated chronically infected rats (Fig. 4), thus, providing evidence of the mitochondrial origin of pathological oxidative stress in chagasic hearts.

PBN treatment of infected rats provided direct evidence of the detrimental effects of oxidative stress and mitochondrial dysfunction in Chagas disease. Oral delivery of PBN (\pm BZ) resulted in preservation of LV function (Online Table 3). It is important to note that beneficial effects of PBN in preserving cardiac function were observed despite no decline in parasite persistence (Fig. 1) and moderate to no change in myocardial inflammatory infiltrate and proinflammatory cytokines in chronically infected hearts (Fig. 5). Instead, PBN-treated/chronically infected rats exhibited a substantial increase in mitochondrial function as evidenced by improved complex activities and ATP synthesis and decreased ROS production (Fig. 4). The decrease in mitochondrial ROS levels was associated with a significant decrease in the myocardial accumulation of MDA, HNE, and carbonyls adducts in PBN-treated/chronically infected rats (Fig. 3). The maximal benefits were obtained when rats were treated with PBN and BZ in combination; thus, suggesting that combinatorial therapies, including antiparasitic drugs and antioxidants, will be effective in preserving the LV function in chagasic hearts.

Cardiac hypertrophy is a key phenotype of the failing heart and a major independent risk factor predictive of cardiovascular mortality and morbidity. In both experimental models and human chagasic patients, clinical evolution of heart failure is preceded by anatomopathological changes that include ventricular thickness and interstitial fibrosis, followed by ventricular dilation and reduced contractility (27). The re-expression of fetal genes (*ANP*, *BNP*, α_{sk} actin, and β -MHC) is a hallmark of hypertrophic remodeling, and a considerable body of evidence shows the redox regulation of remodeling responses in cardiac diseases of various etiologies (28). Besides ROS, experimental studies have shown that the inflammatory cytokines, tumor necrosis factor- α , interleukin-1 β , and monocyte chemoattractant protein 1, also promote myocardial hypertrophy and contribute to the development and progression of heart failure (29). Our observation of a decrease in the expression of hypertrophic markers and collagen deposition in response to PBN treatment (\pm BZ) (Fig. 6) suggests that ROS signals hypertrophic remodeling in chagasic myocardium. The role of ROS of mitochondrial, but not of inflammatory, origin in signaling hypertrophy in chagasic hearts is provided by the observation that NOX and MPO, the classic mediators of inflammatory ROS, were equally depressed in BZ- and PBN-treated rats (Fig. 5), and yet hypertrophic phenotype was depressed in PBN-treated rats only (Fig. 6). Further studies would identify whether inflammatory cytokines synergistically enhance the ROS-mediated signaling cascades involved in activation of hypertrophic responses in chagasic hearts.

Study limitations. We acknowledge the limitations of the rodent model system used in the current study, and the short time-period during which animals were monitored. Further long-term studies are required in larger mammals (e.g. dogs, baboons) that more accurately mimic the human

symptoms and progression of Chagas disease to validate the potential benefits of adjunct therapies in preventing chronic evolution of Chagas disease.

Conclusions

We have shown that complex inflammatory and oxidative processes in the heart contribute to the evolution of chronic hypertrophy and LV dysfunction in Chagas disease. Specifically, PBN treatment enhanced the antioxidant/oxidant balance, preserved the mitochondrial respiratory chain activity and ATP synthesis, and averted the ROS-induced hypertrophy and LV dysfunction in chronically infected rats. BZ treatment was effective in controlling parasite persistence and associated inflammation, but the feedback cycle of mitochondrial dysfunction and oxidative stress persisted, and, therefore, LV function was not significantly improved in BZ-treated/chronically infected rats. Thus, we propose that a combination of antioxidants capable of modulating or delaying the onset of oxidative insult and mitochondrial deficiencies and anti-parasite drugs capable of abolishing parasites and parasite-associated inflammation in the myocardium would prove beneficial in preventing cardiac pathology and loss of LV function in Chagas disease.

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Key Words: antioxidant ■ benznidazole ■ Chagas disease ■ mitochondrial oxidative stress ■ *Trypanosoma cruzi*.

APPENDIX

For details of the methods and supplemental figures and tables, please see the online version of this article.